DNA Double-strand Break Signaling Is a Therapeutic Target in Head and Neck Cancer

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Abstract. *Background:* Head and neck cancer (HNC) is common worldwide. Given poor outcomes for patients with HNC, research into targeted therapies is needed. Ataxia telangiectasia mutated (ATM) is a DNA damage kinase which is activated by double-strand DNA breaks. We tested the effects of a novel ATM inhibitor on HNC cell lines and xenografts. *Materials and Methods:* p53-Binding protein 1 and phosphorylated ATM were localized in cultured cells by immunofluorescence microscopy. Protein expression was determined by western blot. Tumor xenografts were established by injecting HNC lines into immunocompromised mice. Tumor sections were characterized by immunohistochemistry. Apoptotic cells were determined by terminal transferase-mediated dUTP nick-end labeling assay. *Results:* ATM inhibition increased double-strand DNA breaks at replication foci in HNC cell lines. ATM inhibition affected cell-cycle regulatory protein expression, blocked cell-cycle progression at the G₂/M phase and resulted in apoptosis. *Conclusion:* ATM inhibition may be therapeutically useful in treating HNC.

Head and neck cancer (HNC) is common worldwide, with more than 600,000 cases each year (1). HNC is associated with tobacco and alcohol use, and in recent decades human papillomavirus (HPV) infection has been associated with a subset of cancer of the oropharynx (2). The majority of

patients with head and neck squamous cell carcinoma presents with advanced tumors and lymph node metastasis, and recurrence after treatment is a frequent clinical problem (2). Standard treatment for advanced tumors consists of concurrent chemoradiation followed by surgery.

A few targeted therapies have been approved for treatment of HNC. The anti-epidermal growth factor receptor antibody cetuximab was approved for recurrent and metastatic HNC (3, 4). More recently, immune checkpoint inhibitors such as the programmed cell death protein 1 antibodies nivolumab and pembrolizumab were approved for patients with recurrent or metastatic disease previously treated with platinum chemotherapy (2, 5). Given poor clinical outcomes for many patients with HNC, additional research into targeted therapies is needed.

Ataxia telangiectasia mutated (ATM) is a DNA damage kinase which is activated by double-strand DNA breaks, and phosphorylates numerous downstream protein targets (6). When activated, ATM normally arrests the cell cycle to allow DNA repair to occur. Activated ATM is associated with double-strand breaks during DNA synthesis, which may be the result of replication stress. ATM inhibitors may kill cancer cells due to the persistence of unrepaired double-strand breaks leading to apoptosis.

ATM inhibitors have largely been unsuccessful in the cancer clinic. AZD1390 is a first-in-class orally available ATM inhibitor with greater than 10⁴-fold selectivity over other phosphatidylinositol 3'-kinases (7). We tested the effects of novel ATM inhibitor AZD1390 on HNC cell lines and xenografts.

Materials and Methods

Cell culture. Human HPV- SCC lines SCC9, SCC15, SCC25 and SCC71, and HPV+ lines SCC152 and SCC154 were purchased from the American Type Culture Collection (Manassas, VA, USA);

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SCC90 and SCC104 were purchased from MilliporeSigma (Burlington, MA, USA). Cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 40 µg/ml gentamicin at 37°C in a humidified atmosphere of 5% CO₂. All cell lines were negative for mycoplasma. Triplicate cultures were treated with 0.2-5 µM ATM inhibitor AZD1390 (Selleck Chemicals, Houston, TX, USA) for 1-5 days. Cell death was monitored by trypan blue exclusion analysis. The positive cell fraction was determined using a quantitative digital pathology image analysis system (Vectra 3; Akoya Biosciences, Marlborough, MA, USA).

Antibodies. The following antibodies were used in this study: ATM (AB201022), phospho-checkpoint kinase 2 (pCHK2^{T68}) (AB85743), human papillomavirus (HPV) virion (HPV L1) (AB25275), cyclin-dependent kinase inhibitor 2A (p16^{INK4A}) (AB54210), p-histone H3 (AB47297), anti-IgG/Alexafluor 488 (AB650077), and anti-IgG/Alexafluor 555 (AB150074) from Abcam (Waltham, MA, USA); checkpoint kinase 2 (CHK2) (611570), cyclin-dependent kinase 1 (CDK1) (C12720), CDK2 (C18520), cyclin D1 (C20320) and p53 (554147) from BD Biosciences (Franklin Lakes, NJ, USA); β-actin (4970S) from Cell Signaling (Danvers, MA, USA); p53 binding protein 1 (53BP1) (NB100-304) from Novus (Centennial, CO, USA); pATM^{S1981} (SC-47739), cyclin B1 (SC-752), CDK4 (SC-601), CDK6 (SC-177), proliferating cell nuclear antigen (PCNA) (SC-7907) from Santa Cruz Biotechnology (Dallas, TX, USA) and keratin 10 antibody (K10) (C-7284) from Sigma (St. Louis, MO, USA).

Mouse procedures. Mouse procedures were approved by the Institutional Animal Care Committee (19-027) and performed in accordance with ARRIVE guidelines (8). Immunocompromised NU/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA; n=10 for each cell line). This sample size would allow detection of greater than 25% reduction in tumor volume due to treatment with alpha error =0.05 and power =0.8. Two-month-old male and female mice were subcutaneously injected with 10⁶ cells from each line suspended in 0.1 ml Matrigel. Mice with 100 mm³ tumors were randomly assigned to 50 mg/kg AZD1390 or vehicle-treated groups. AZD1390 or vehicle was administered by oral gavage. Mice were dosed once daily for 5 days. Tumor volume was recorded daily for each animal. Mice underwent complete necropsy after day 5. Tumors were dissected and fixed in 4% buffered formaldehyde. Researchers were blinded to identities of control and experimental samples.

Histopathology, immunofluorescence, and immunohistochemistry. In cell culture experiments, cells were treated with 0.2 µM AZD1390 or vehicle for 16 hours and fixed in 2% formaldehyde for 10 minutes, permeabilized with 70% ethanol for 10 minutes, washed in phosphate-buffered saline (PBS), and incubated with 1:100 dilution of antibodies to 53BP1, pATM, and PCNA. After washing, cells were incubated with anti-IgG secondary antibodies conjugated to AlexaFluor 488 or AlexaFluor 555. After washing, slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and coverslipped with anti-fade mounting medium. For histopathology experiments, formalin-fixed tumors were dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Tumor sections were deparaffinized and stained with hematoxylin and eosin. For immunofluorescence experiments, tumor sections were rehydrated in PBS (pH 7.4), blocked with 10% normal serum, and incubated with HPV L1 or keratin 10 antibodies overnight at room temperature. After washing, sections

were incubated with anti-IgG secondary antibodies conjugated to AlexaFluor 488 or AlexaFluor 555. After washing, slides were counterstained with DAPI and coverslipped with anti-fade mounting medium. Sections were visualized using fluorescence microscopy (Zeiss LSM 710 META; Zeiss, White Plains, NY, USA). For immunohistochemical analysis, sections were rehydrated in PBS and blocked using 10% normal serum and incubated with 1:50 dilution of pATM, PCNA, p16^{INK4A}, or pCHK2 antibodies overnight at room temperature. After washing, sections were incubated with biotinylated secondary antibody at room temperature for 10 min. After additional washing, sections were incubated with streptavidin-conjugated horseradish peroxidase for 10 min at room temperature. Antigen-antibody complexes were detected by incubation with peroxide substrate solution containing aminoethylcarbazole chromogen. The positive cell fraction was determined using a quantitative digital pathology image analysis system (Vectra 3; Akoya Biosciences).

Transmission electron microscopy. To determine if tumor xenografts produce HPV virions, tumor xenografts were fixed in phosphate-buffered 2% glutaraldehyde, postfixed in phosphate-buffered 1% osmium tetroxide, dehydrated in acetone series, infiltrated in propylene oxide/resin, and embedded in Epon resin. Sections of 0.1 mm were stained with uranyl acetate and lead citrate and imaged using a JEOL JEM-1220 electron microscope (JEOL, Peabody, MA, USA).

Cell death analysis by terminal transferase-mediated dUTP nick-end labeling (TUNEL) assay. Tumor sections or human SCC cells treated with DMSO or 0.2 µM AZD1390 for 16 hours were fixed, permeabilized, and incubated with terminal deoxynucleotidyl transferase and dUTP-fluorescein for 1 h at 37°C according to manufacturer's recommendations (Roche Applied Sciences, Indianapolis, IN, USA). After washing, apoptotic cells were visualized by fluorescence microscopy. The fluorescent cell fraction was determined using a quantitative digital pathology image analysis system (Vectra 3; Akoya Biosciences).

Western blot. Protein was extracted from human SCC lines treated with DMSO or 0.2 µM AZD1390 for 16 hours in 1× Laemmli buffer (0.1 M Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 0.1 M dithiothreitol, 10% glycerol, 0.0025% bromophenol blue). 75 µg total cellular protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were electroblotted to polyvinylidene difluoride membranes. Blots were incubated with antibodies to pATM^{Ser1981}, ATM, CHK2, p53, cyclin B, cyclin D, CDK1, CDK2, CDK4, CDK6, p-histone H3^{S10}, or β-actin for 16 hours at 4°C. After washing, blots were incubated for 30 minutes at room temperature with anti-IgG secondary antibody conjugated to horseradish peroxidase. Bands were visualized by the enhanced chemiluminescence method, normalized to β-actin expression, and quantitated by image analysis software (Silk Scientific, Orem, UT, USA).

Fluorescence-activated cell sorting. Human SCC lines treated with DMSO or 0.2 µM AZD1390 for 16 hours were dissociated by trypsinization, washed in PBS, fixed in 70% ethanol, stained with propidium iodide, washed in PBS, and subjected to cell-cycle analysis by fluorescence-activated cell sorting.

Statistical analysis. Parametric data were analyzed by unpaired two-sided *t*-test. Non-parametric data were analyzed by Fisher exact test.

Figure 1. Inhibition of ataxia telangiectasia mutated (ATM) increases double-strand DNA breaks at replication foci and head and neck cancer cell death in vitro. A: phospho (p)ATM expression at proliferating cell nuclear antigen (PCNA⁺) DNA replication foci in dimethyl sulfoxide (DMSO)-treated and in AZD1390-treated SCC9 and SCC152 cells as shown by immunofluorescence microscopy. B: p53-binding protein 1 (53BP1) expression at PCNA⁺ DNA replication foci in DMSO-treated and in AZD1390-treated SCC9 and SCC152 cells as shown by immunofluorescence microscopy. Programmed cell death in DMSO-treated and in AZD1390-treated SCC9 and SCC152 cells as shown by terminal transferase-mediated dUTP nick-end labeling (TUNEL) analysis. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Scale bar=10 μ m. C: Quantitation of 53BP1⁺ and TUNEL⁺ cells in SCC9 and SCC152 lines treated with DMSO or AZD1390. Error bars indicate SEM. Experiments were performed three times with similar results.

Figure 2. Inhibition of ataxia telangiectasia mutated (ATM) reduces expression of cell cycle-regulatory proteins in head and neck cancer (HNC) cell lines. ATM, phospho (p)-ATM, checkpoint kinase 2 (CHK2), pCHK2, p53, cyclin B, cyclin D, cyclin dependent kinase 1 (CDK1), CDK2, CDK4, and CDK6 expression in eight HNC cell lines treated with dimethyl sulfoxide (DMSO) or AZD1390 is shown by western blot. β-Actin expression was used to control for protein loading in each lane. Experiments were performed three times with similar results.

Results

A novel ATM inhibitor increased double-strand DNA breaks and impaired survival of HNC cell lines. Previous ATM inhibitors have been largely unsuccessful in the cancer clinic. To determine if use of a novel ATM inhibitor AZD1390 results in increased double-strand breaks in HNC cell lines, we examined pATM and 53BP1 expression at PCNA⁺ replication foci in HPV⁻ SCC9 and HPV⁺ SCC152 cells treated with DMSO or 0.2 μM AZD1390 for 16 hours. pATM colocalized at PCNA⁺ DNA replication foci in DMSO and AZD1390-treated HPV⁺ and HPV⁻ cells (98%; Figure

1A). ATM inhibition increased 53BP1⁺ co-localization at DNA replication foci in both HPV⁻ (70% vs. 51%; $p < 0.02$) and HPV⁺ (75% vs. 58%; $p < 0.04$) HNC cell lines (Figure 1B and C). ATM inhibition increased the TUNEL⁺ cell fraction in HPV⁻ (77% vs. 0.2%; $p < 0.0001$) and HPV⁺ (86% vs. 0.1%; $p < 0.00002$) HNC cell lines (Figure 1D). Similar results were observed in additional HPV⁻ and HPV⁺ HNC cell lines (data not shown). These results indicate that ATM inhibition results in increased double-strand breaks in HPV⁻ and HPV⁺ HNC cell lines. Increased double-strand DNA breaks correlated with programmed cell death in HPV⁻ and HPV⁺ HNC lines.

Figure 3. Quantitation of phospho-ataxia telangiectasia mutated (ATM) (A), cyclin B (B), cyclin-dependent kinase 1 (CDK1) (C), CDK2 (D), and CDK6 (E) protein expression in indicated SCC cell lines treated with vehicle or AZD1390. Error bars indicate the standard error of the mean. Asterisks indicate statistically significant differences from control cells.

ATM inhibition regulates expression of cell-cycle proteins in HNC cell lines. To determine if ATM inhibition affected expression of cell-cycle regulatory proteins in HNC, we examined expression of these proteins by western blot. pATM expression was reduced 2- to 8-fold by AZD1390 treatment of HPV⁻ SCC9, SCC15, SCC25 and SCC71, and HPV⁺ SCC90, SCC104 and SCC152 cell lines ($p < 0.05$; Figure 2 and Figure 3). pCHK2 expression was detected in HPV⁻ and HPV⁺ cell lines and was unaffected by AZD1390 treatment, likely due to phosphorylation by other DNA-damage kinases (see Discussion). Expression of G₂/M-phase cyclin B was inhibited 3-fold in HPV⁻ SCC15 and 4-fold in HPV⁻ SCC25 and SCC71 and HPV⁺ SCC152 cell lines by AZD1390 treatment ($p < 0.01$; Figure 2 and Figure 3). Expression of the G₂/M phase CDK1 was inhibited by 2-fold in HPV⁺ SCC90 and SCC104 cell lines by AZD1390 treatment ($p < 0.05$; Figure 2 and Figure 3). Expression of S-phase CDK2 expression was inhibited by 2-fold in HPV⁻ SCC15, SCC25 and SCC71, and HPV⁺ SCC104, SCC154 cells, and 3-fold in HPV⁺ SCC90 cells by AZD1390 treatment ($p < 0.02$; Figure 2 and Figure 3). Expression of G₁-phase CDK6 was inhibited by 2-fold in HPV⁺ SCC104 and SCC154 cell lines by AZD1390 treatment ($p < 0.05$; Figure 2 and Figure 3). Expression of G₁-phase CDK4 was not significantly affected by AZD1390 treatment. Expression of p53 and G₁-phase cyclin D1 was detected only in HPV⁻ SCC9 cells and was not affected by AZD1390 treatment. p53 is expressed at low levels in most cells due to MDM2 proto-oncogene ubiquitin ligase and loss of heterozygosity in cancer cells, although some cancer-associated p53 mutants exhibit increased protein stability (9). Cyclin D1 also is overexpressed in some HNCs (10). These results indicate that ATM inhibition regulates expression of S and G₂/M-phase cell cycle-related proteins in HNC cell lines.

ATM inhibition regulates cell-cycle progression in HNC cell lines. To determine if reduced expression of G₂/M-phase proteins affects cell-cycle progression, we profiled vehicle and AZD1390-treated HNC cell lines by fluorescence-activated cell sorting. AZD1390 treatment reduced G₂/M phase HPV⁻ SCC9 (4.2% vs. 18.3%; $p < 0.007$; Figure 4A) and HPV⁺ SCC152 (6.7% vs. 16.8%; $p < 0.01$; Figure 4B and C) cell fractions. Similar results were observed in additional HPV⁻ and HPV⁺ cell lines (data not shown). Expression of the S-phase protein phospho-histone H3 was increased 9-fold by AZD1390 treatment (Figure 4C), indicating cell-cycle block at the G₂/M-phase checkpoint. These results indicate ATM inhibition blocks cell-cycle progression at G₂/M phase in HPV⁻ and HPV⁺ HNC cell lines, likely due to accumulation of double-strand DNA breaks.

ATM inhibition induces cell death in HNC lines. To confirm if ATM inhibition induces cell death in HPV⁻ and HPV⁺ HNC cell lines, we performed *in vitro* time course and dose-response

experiments using trypan blue exclusion analysis. AZD1390 treatment induced time- and dose-dependent cell death in HPV⁻ SCC9 (Figure 5A) and HPV⁺ SCC152 (Figure 5B) cell lines, with 5 μ M AZD1390 resulting in no viable cells by 4 days of treatment. Similar results were observed in additional HPV⁻ and HPV⁺ cell lines (data not shown). There were no statistically significant differences in cell death between HPV⁻ and HPV⁺ cell lines. These results indicate that ATM inhibition induces cell death in HPV⁻ and HPV⁺ HNC cell lines *in vitro*.

ATM inhibition inhibits growth of HNC xenografts. To determine the effects of ATM inhibition on HNC growth *in vivo*, we transplanted HPV⁻ and HPV⁺ cancer cell lines subcutaneously in NU/J mice. HPV⁺ xenografts expressed high levels of the HPV capsid protein L1 as shown by immunofluorescence microscopy which was not observed in HPV⁻ transplanted tumors (Figure 6A and C). HPV virions were observed in xenografts derived from HPV⁺ HNC cell lines as shown by transmission electron microscopy (Figure 6B and D). This is a novel result which has not been previously reported. We treated mice with transplanted tumors with vehicle or AZD1390. AZD1390 treatment inhibited tumor growth by 87% in HPV⁻ SCC9 and 82% in HPV⁺ SCC152 transplanted mice ($p < 0.0008$; Figure 6E). Similar results were observed in additional HPV⁻ and HPV⁺ cell lines (data not shown). These results indicate that AZD1390 inhibits growth of HPV⁻ and HPV⁺ HNC cell lines *in vivo*.

ATM inhibition inhibits proliferation and induces cell death in HNC xenografts. We examined AZD1390- and vehicle-treated HPV⁻ and HPV⁺ transplanted HNC by histopathology, immunohistochemistry, and TUNEL analysis. Vehicle-treated mice bearing transplanted HPV⁻ and HPV⁺ tumors were highly cellular and poorly differentiated (Figure 7A). Cells exhibited high nuclear/cytoplasmic ratio characteristic of proliferating basal cells observed in human HNC. AZD1390 treatment resulted in small hypocellular tumors with fragmented nuclei characteristic of programmed cell death (Figure 7A). HPV⁺ tumors contained a high p16^{INK4A}-positive cell fraction (74% vs. 2%; $p < 0.001$) compared to HPV⁻ tumors (Figure 7B), which is characteristic of human HNC. p16^{INK4A} expression was not detected in tumors from AZD1390-treated mice due to extensive cell death (Figure 7B). pATM was not detectable by immunohistochemistry in transplanted HPV⁻ and HPV⁺ HNC (Figure 7C). However, expression of downstream target pCHK2 was increased in HPV⁺ transplanted tumors (28% vs. 1%; $p < 0.003$) compared to HPV⁻ tumors (Figure 7D). pCHK2 expression was not detected in tumors from AZD1390-treated mice due to extensive cell death (Figure 7D). The PCNA⁺ proliferating cell fraction was high in both HPV⁻ and HPV⁺ transplanted tumors (59% vs. 49%; Figure 7E). PCNA expression was not detected in tumors from

Figure 4. Inhibition of ataxia telangiectasia mutated (ATM) blocks head and neck cancer (HNC) cell lines at G₂/M phase of the cell cycle. Cell-cycle analysis of dimethyl sulfoxide (DMSO)-treated and AZD1390-treated SCC9 (A) and SCC152 (B) cells is shown by fluorescence activated cell sorting. C: Increased phospho-histone H3 expression in SCC9 and SCC152 cells treated with AZD1390 compared to DMSO-treated cultures is shown by western blot. β-Actin expression was used to control for protein loading in each lane. Experiments were performed three times with similar results.

Figure 5. Ataxia telangiectasia mutated (ATM) inhibition induces death of head and neck cancer cell lines *in vitro*. Time course and dose response of SCC9 (A) and SCC152 (B) cell lines to AZD1390 is shown by trypan blue exclusion analysis. Experiments were performed three times with similar results.

AZD1390-treated mice due to extensive cell death (Figure 7E). The TUNEL⁺ cell fraction was increased in AZD1390-treated HPV⁻ and HPV⁺ (21% vs. 0.3%; $p < 0.006$; Figure 7F) tumors compared to vehicle-treated controls, although

this is likely underestimated due to extensive nuclear fragmentation. These results indicate that AZD1390 treatment inhibits proliferation and induces cell death in transplanted HPV⁻ and HPV⁺ HNC *in vivo*.

Figure 6. Head and neck cancer (HNC) xenografts produce human papillomavirus (HPV) virions. A: HPV L1 and keratin 10 (K10) expression is shown in an SCC9 xenograft under immunofluorescence microscopy. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Scale bar=10 μ m. B: SCC9 xenograft is shown by transmission electron microscopy. Scale bar=50 nm. C: HPV L1 and K10 expression is shown in an SCC152 xenograft under immunofluorescence microscopy. D: HPV virion (arrow) in SCC152 xenograft as seen under transmission electron microscopy. E: ATM inhibition prevents growth of transplanted HNC cell lines in vivo. Tumor volume was measured in mice transplanted with SCC9 or SCC152 cells and treated with vehicle or AZD1390 for 5 days. Error bars indicate the standard error of the mean. Experiments were performed three times with similar results.

Discussion

Our data demonstrated that ATM co-localizes at PCNA⁺ DNA replication foci in both HPV⁻ and HPV⁺ human SCC lines. Previous study suggested that ATM activation at sites of DNA synthesis is the result of replication stress (11). ATM

inhibition prevents double-strand DNA break repair in human SCC lines as indicated by increased 53BP1 foci. ATM has a well characterized role in repair of double-strand DNA breaks (12). ATM inhibition in human HNC lines prevents progression into G₂/M phase with associated changes in cell cycle-regulatory protein expression. ATM also regulates the

Figure 7. Inhibition of ataxia telangiectasia mutated (ATM) induces death of head and neck cancer cell lines *in vivo*. A: Histopathology of SCC9 and SCC152 transplanted tumors from vehicle-treated and AZD1390-treated mice as shown by hematoxylin and eosin staining. Immunohistochemical expression of cyclin-dependent kinase inhibitor 2A (p16^{INK4A}) (B), phospho-(p)ATM (C), phospho-checkpoint kinase 2 (pCHK2) (D), and proliferating cell nuclear antigen (PCNA) (E) expression in SCC9 and SCC152 transplanted tumors from vehicle-treated and AZD1390-treated mice. Nuclei were counterstained with hematoxylin. F: Programmed cell death in SCC9 and SCC152 transplanted tumors from vehicle-treated and AZD1390-treated mice as shown by terminal transferase-mediated dUTP nick-end labeling (TUNEL) analysis. Nuclei were counterstained with 4',6-diamidino-2-phenylindole. Scale bar=10 μ m. Experiments were performed three times with similar results.

DNA-damage checkpoint at this cell-cycle phase (13). In addition to ATM, CHK2 can be phosphorylated by other DNA damage kinases such as DNA-dependent protein kinase and polo-like kinase 3 (14, 15). Our data demonstrated that ATM inhibition has potent cell cycle-inhibitory effects which correlated with apoptosis in both HPV⁻ and HPV⁺ human SCC lines *in vitro* and *in vivo*. If double-strand DNA breaks are not repaired, cells may undergo programmed cell death to prevent replication of damaged DNA. DNA-damage induction is a common mechanism by which chemotherapeutic agents exert their anti-tumor effects.

Our data demonstrated that p16^{INK4A} is expressed by transplanted HPV⁺ SCC lines *in vivo*. p16^{INK4A} is a diagnostic biomarker for HPV⁺ oropharyngeal cancer, and was shown to inhibit homologous recombination-mediated DNA repair in HPV⁺ oropharyngeal cancer cell lines (16).

HPV research has been impeded by the difficulty of producing virions in monolayer culture (17). Recombinant L1 protein assembles into virus-like shells *in vitro* (18), and HPV has been propagated in epidermal three-dimensional organotypic cultures (19, 20) and in epidermal transplants to the kidney capsule of severe combined immunodeficient mice (21). Our HPV virion model reported in this article has the advantage of technical simplicity and reproducibility using publicly available cell lines. Plasma HPV DNA was predictive of oropharyngeal cancer recurrence in a recent clinical study (22), although it was not clear whether the source of this DNA was tumor mediated virus production or cancer cell death.

Our study demonstrated that DNA double-strand break signaling is a target for therapy in both HPV⁺ and HPV⁻ disease. HPV⁺ HNC has better prognosis than HPV⁻ tumors, which is likely due to their lower DNA mutational burden (23). Our study indicates that ATM inhibition is an effective therapy for both types of HNC, likely due to its cell cycle-inhibitory effect which induces apoptosis. Future studies will determine infectivity of tumor-produced HPV and its possible role in HNC progression, and reconcile HPV-induced genetic changes with clinical prognosis.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

JW, KJG, and RDB performed experiments. CEP, AS, and DLC analyzed data and wrote the article.

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